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EFFECTIVENESS OF THE CHEMICAL STABILIZERS OF *TALAROMYCES FLAVUS* IN BIOLOGICAL CONTROL OF TOMATO AND GREENHOUSE CUCUMBER PYTHIUM ROOT ROT DISEASE

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ABSTRACT

In Iran, considerable research have been already carried out, about the mass production of the different Talaromyces flavus isolates and their efficacy in controlling some important soil borne diseases, in several crops have been also investigated. For the application of these biological products in the fields, the technical science related to their production should be assigned to the producer companies. In this study, according to the specific metabolites, related to these mechanisms, the stabilizers of these metabolites were used in developing bioformulations, containing T. flavus isolates. Based on the previous studies, the effective substrate in terms of the sporulation and the efficacy of T. flavus isolates, was rice bran-peat moss. Results showed that, two T. Flavors bioformulations related to dicycloserine and carboxymethyl cellulose, bioformulation containing carboxymethyl cellulose was more effective, than dicycloserine in controlling above-mentioned pathogenic agents, for treatments related to tomatoes; however, this position was reversely for greenhouse treatments.

KEYWORDS: Stabilizer, Antagonistic Fungus & Metabolite

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Original Article

INTRODUCTION

Soil-borne diseases, such as Verticillium wilt, Fusarium wilt, Pythium root rot, and seedling damping-off, are common in most crops and greenhouse products, including greenhouse tomato and cucumber, in Iran (Heydari and Pesarakly, 2010, Ghaderi, 2011; Sharzehi *et al*., 2011). The fungus *Talaromyces flavus*, as an antagonist of the agents of soil-borne fungus, including *Fusarium oxysporum*, *Verticillium albo-atrum*, *Verticillium dahliae*, and *Rhizoctonia solani*, has been widely discussed in the literature abroad (Madi *et al*., 1992; Madi *et al*., 1997; Duo-Chuan *et al*., 2005; Haggag *et al*., 2006; Shikhul Ashraf and Ahmad Khan, 2007). So, the various isolates of the antagonistic fungi were attempted to be separated into the major areas of cultivation of some crops in Iran.

After a lot of lab trials and greenhouse research, on the antagonistic effects of the isolates on the above-mentioned pathogens, and after introducing the most effective isolates of each product (in terms of pathogen control), the biological fungicides affected by the different isolates of *T. Flavus,* were prepared separately to control the above-mentioned pathogens of each product. To use these fungicides on a large scale, they must be mass-produced and the technical knowledge of their production must be transferred to the manufacturers. In this regard, the issues of interest for these manufacturers, it could be the marketing and commercialization of the fungicides

(Alimi *et al*., 2006; Husen *et al*., 2007; Kaewchai *et al*., 2009; Pereira *et al*., 2009). According to the existing research, the efficiency and sustainability of these fungicides are the most important factors, that affect their marketing and commercialization (Kaewchai *et al*., 2009; Mukhopadhyay and Maiti, 2009; Ghaderi-Daneshmand *et al*., 2012).

Previous complementary studies have determined the antagonistic mechanisms of many different isolates of *T. Flavus,* on some pathogens of the above-mentioned products and their specific metabolites. Using the research done abroad, on the introduction of organic and inorganic stabilizer compounds for metabolites (Yu and Chang, 1987; Cimarelli *et al*., 2001; Matos *et al*., 2012), this study tried to optimize the *T. flavus* inoculates, to strengthen two important features. These are sustained (durability before use in the farm) and efficient (the ability to control the disease in the form). By studying the efficiency of *T. flavus* inoculums, in controlling some important soil-borne pathogens in greenhouse cucumber and tomato products, with the use of different stabilizers in this research, the answer to the following questions became clear:

- To prepare *T. flavus* inoculum, what is the most effective stabilizer compound, in terms of biological control of Pythium root rot in tomato?
- To prepare *T. flavus* inoculum, what is the most effective stabilizer compound, in terms of biological control of Pythium root rot in greenhouse cucumber?
- Are effective stabilizers, specific to the above two products, and/or can a common stabilizer for both products to be obtained?

In recent decades, many reports have been presented, on the preparation of biological fungicides, using solid substrates and their optimization in various stages of production (Pascual *et al*., 1999; Budge and Whipps, 2001; Schuster and Schmoll, 2010; Caramez *et al*., 2012; Sargin *et al*., 2013). For example, Pascual *et al.* (1999) provided a solid biological fungicide for wheat, affected by the fungus *Epicoccum nigrum.* After reviewing the alcoholic solution containing glycerol, mannitol, and arabitol, on the sporulation of this fungus, the highest sporulation-significant increase was reported in glycerol. Also, to increase the efficiency of the biological fungicide, by *Trichoderma harzianum* EGE-K38, Sargin *et al.* (2013), compared various methods to dry the fungicide. The research results also showed that, the use of compounds containing minerals like manganese, iron, zinc, and phosphorus, to produce bio-fertilizers increases their sustainability (Vasane and Kothari, 2008; Lee and Lee, 2009).

So far, biological fungicides, such as Ketomium, derived from *Chaetomium globosum* and *C. cupreum*, Promote, derived from *T. harzianum* and *T. viride*, SoilGard, derived from *Gliocladium virens*, Trichodex, derived from T. harzianum, Pisolithus tinctonus, and Glomus intraradices, Trichodermin, derived from *T. harzianum*, and Protus WG, derived from *T. flavus*, have been commercially registered in the country (Merwel *et al*., 1974; Koch, 1999; Kaewchai *et al*., 2009).

In Iran, greenhouse and farm studies were conducted using *T. Flavus,* on the biological control of Verticillium wilt in cotton and potato, Fusarium wilt in tomato, and damping-off of sugar beet seedlings. The results showed that, in addition to reducing the disease index significantly, the fungus also caused a significant increase in earliness and yield (Naraghi *et al*., 2014a and b; Farhang Niya *et al*., 2015). Also, the results of the greenhouse experiment, on the biological control of Verticillium wilt of greenhouse potato, tomato, and cucumber, due to *V. albo-atrum* by the different isolates of *T. flavus* showed that, the isolates were significantly effective in reducing the disease index and increasing growth traits

like root length, stem length, height, and the wet and dry weights of the plants (Naraghi *et al.*, 2010a, b and c).

MATERIALS AND METHODS

Preparing the Treatments Derived from the Isolates of the Fungus *T. Flavus* to be Used in a Greenhouse

Select the Most Effective *T. Flavus* Isolates

Following previous research (Naraghi *et al.*, 2010b and c), the most effective isolates of *T. Flavus,* in terms of control of some important soil-borne diseases in greenhouse tomato and cucumber, were selected in this stage. These included TF-To-V-24 (isolate 24 obtained from Varamin tomato farms' soil) and TF-Cu-V-60 (isolate 60 obtained from Varamin cucumber greenhouse soil).

Prepare the *T. Flavus* Bio formulations

To prepare the applied bio formulations of the relevant isolates (TF-To-V-24 and TF-Cu-V-60), the modified method of Naraghi *et al.* (2010c) was used. So, rice bran was soaked in water at 30–35°C, for 24 hours. Then, it was spread on a large filter paper and dried. In the next stage, 200g rice bran and 50g peat soil were washed and sterilized in cellophane bags, in the autoclave (1 atmosphere pressure, 120°C temperature for 15 minutes). In the next stage, a suspension containing 20ml sterile distilled water and four 1cm pieces of 10-day medium of the relevant isolate were poured into cellophane bags, to prepare the bioformulation for each isolate. Then, the stabilizer compounds, including amino phenol, D-cycloserine, magnesium sulphate, carboxymethyl cellulose, and sodium nitrate, were added according to the treatment, based on the addition of supplements to the substrates (10ml solution supplement of 20g per litre to 250g per substrate). For the isolates' growth, the cellophane bags were put in the incubator for a month and a half to two months at 30°C. If the content had to be dried during this period, 20ml distilled water was added to create moisture. Then, the content of each cellophane bag was put on filter paper to dry. It was then added to soil as bio formulation used in the greenhouse.

The bio formulation was added to the soil according to the 2×10^7 spore-per-gram rule. The inoculum was determined based on the treatment and its amount was calculated by the number of spores per gram by the haemocytometer (Aziz *et al.*, 1997).

Prepare the Treatments Derived from Pathogenic Isolates to be Used in a Greenhouse

Prepare Pathogenic Isolates

At this stage, for *Pythium aphanidermatum*, the isolate existing in Varamin Research Laboratory was used. This isolate (PA-Cu-PV-1) was taken from the root of cucumber in Varamin. In this study, it was used to prepare the pathogenic inoculum of *P. aphanidermatum* to treat greenhouse tomato and cucumber.

Prepare and Inoculate the Pathogenic Inoculum

To prepare the inoculum of *P. aphanidermatum* (PA-Cu-PV-1) isolate, the isolate was cultured in 9cm-diameter Petri dishes containing Corn Meal Agar (CMA) medium. After 24 hours, each dish row with the agar medium was used as an inoculum (Brantner and Windels, 1998; Salas *et al.*, 2003). For the inoculation, the soil around the root was removed, the isolate cultured in the medium in 9cm Petri dishes was put beside the root with a sterile scalpel, the pot soil was put on it, and it was irrigated immediately.

Assessing the Antagonistic Ability of the Treatments of *T. Flavus* on Pythium Root Rot in Greenhouse Tomato and Cucumber

At this stage, two experiments were performed separately for both greenhouse tomato and cucumber affected by the *Pythium aphanidermatum* pathogen. Each trial had a randomized complete block design with three replications and seven treatments. Each experiment treatment consisted of *T. flavus* bioformulations affected by the five different stabilizers (amino phenol, D-cycloserine, carboxymethyl cellulose, magnesium sulphate, and sodium nitrate) on healthy and infected control. The final assessment of each experiment was conducted based on a relevant disease index. Also, the data were statistically analyzed using Duncan's multiple range test and the mean classification.

The time and method of assessing the disease were as follows:

The disease was assessed four weeks after inoculation by determining the percentage of the disease intensity on a 1–7 scale, as follows (Altier and Theis, 1995; Panella, 1998):

1= the plant with roots, no signs and healthy

2= the plant with necrotic signs at the end of the main and hairy root

3= the plant with necrotic signs in 25% of the root

4= the plant with necrotic signs in 50% of the root

5= the plant with necrotic signs in 75% of the root

6= the plant with decayed root and green leaves

7= the plant's death

The disease intensity percentage: the number of plants on scale 1+ the number of plants on scale 2+… + number of plants on scale 7×100/ total number of plants ×7

Separating Pathogenic Fungal Factors from Infected Plant Tissues

First, the infected seedling crown and root were washed under tap water for a few minutes. In the next stage, they were disinfected with a solution of commercial bleach (containing 5% sodium hypochlorite) for 10 to 60 seconds. They were then washed thrice in three separate dishes with sterile distilled water and dried on sterilized filter paper and/or sterilized cotton soaked in 95% ethanol by touching the infected area. Then, using a tissue scalpel with patches, they were cut into pieces. The pieces were selected from the healthy and infected tissue boundary and transferred to Petri dishes contain water-agar (WA) medium. The Petri dishes were kept in an incubator at 22–25°C. After being observed for fungal colonies from 12 to 48 hours, each of them was transferred to a PDA medium for identification and subsequent studies. The identification of each fungal pathogen (*V. dahliae*, *F. oxysporum*, *R. solani* and *P. aphanidermatum*) was based on the macroscopic and microscopic specifications according to the available scientific resources (Kim *et al*., 2001; Summerell *et al*., 2003; Gonzales Garcia *et al*., 2006; Postma *et al*., 2001).

RESULTS

The Required Amount of the Bioformulation of *T. Flavus* to be Used in Pot Soil

The amount of *T. flavus* bioformulation (TF-To-V-24 isolate for greenhouse tomato and TF-Cu-V-60 isolate for

greenhouse cucumber) needed in each pot containing 3kg soil was determined based on 2×10^7 colony unit (CFU) per gram of soil (listed in the methodology) and 6×10^9 unit per gram of colony per gram inoculum prepared from *T. flavus* isolates (TF-To-V-24 and TF -Cu-V-60). Thus, each pot of greenhouse tomato and cucumber was treated according to the 10g treatment with bioformulations containing TF-To-V-24 or TF-Cu-V-60 isolates.

The Required Amount of the Pathogenic Inoculum Of *P. Aphanidermatum* to be Used in Pot Soil

The required amount of pathogenic inoculum of P. Aphanidermatum, to be used in a pot containing 3kg soil was determined based on the 10-day cultured P. aphanidermatum using a 9cm-diameter Petri dish (listed in the methodology). Thus, 10-day cultured isolates of PA-Cu-P-V-1 using a 9cm-diameter Petri dish was used in each pot of greenhouse tomato and cucumber.

Assessing the Antagonistic Ability of the Treatments Affected By *T. Flavus* on Pythium Root Rot in Greenhouse Tomato and Cucumber

Tomato Pythium Root Rot

The effect of *T. flavus* bioformulations containing various stabilizers on tomato Pythium root rot was significant at 1% level. The mean comparison results of the various treatments in terms of the disease severity percent showed that all the means were in four statistical groups. All treatments, except for the one containing amino phenol, showed a significant reduction in the disease intensity percentage compared with the infected control. Among these treatments, the lowest disease severity percent was calculated in the treatment containing the sodium nitrate stabilizer. On the other hand, among other treatments with the stabilizers of D-cycloserine, magnesium sulphate, and carboxymethyl cellulose, no statistically significant difference was observed in terms of the disease severity percent at the 1 % level (Table 1)

Table 1: The Mean Comparison of the Disease Severity Percent of Pythium Root Rot in Tomato Plants After Treatments with *T. Flavus* Bioformulations Containing Different Stabilizers under Greenhouse Conditions

Treatment	Disease Severity (%)
T. flavus bioformulation containing amino phenol stabilizer	64.28a**
T. flavus bioformulation containing D-cycloserine stabilizer	42.85b
T. flavus bioformulation containing magnesium sulphate stabilizer	42.85b
T. flavus bioformulation containing carboxymethyl cellulose stabilizer	42.85b
T. flavus bioformulation containing sodium nitrate stabilizer	28.57c
Infected control	64.28a
Healthy control	0.00d

*****: Treatments Marked by the Same Letter (S) are not Significantly Different (P> 0.01)***

Greenhouse Cucumber Pythium Root Rot

The effect of *T. flavus* bioformulations containing various stabilizers on greenhouse cucumber Pythium root rot was significant at 1% level. The mean comparison results in various treatments, in terms of the disease severity percent showed that all the means were in four statistical groups. All treatments, except for the one containing amino phenol and magnesium sulphate, showed a significant reduction in the disease intensity compared with the infected control. Among these treatments, the lowest disease severity percent was calculated in the treatment containing sodium nitrate stabilizer. Also, among two other treatments related to stabilizers of D-cycloserine and carboxymethyl cellulose, no statistically significant difference was observed in terms of the disease severity percent at the 1 % level (Table 2).

Table 2: The Mean Comparison of the Disease Severity Percent of Pythium Root Rot in Greenhouse Cucumber Plants after Treatments with *T. Flavus* Bioformulations Containing Different Stabilizers under Greenhouse Conditions

Treatment	Disease Severity (%)
T. flavus bioformulation containing amino phenol stabilizer	100.00a**
T. flavus bioformulation containing D-cycloserine stabilizer	71.42b
Table 1: Contd.,	
T. flavus bioformulation containing magnesium sulphate stabilizer	100.00a
T. flavus bioformulation containing carboxymethyl cellulose stabilizer	78.56b
T. flavus bioformulation containing sodium nitrate stabilizer	35.71c
The infected are controlled	100.00a
Healthy control	0.00d

****: Treatments Marked by the Same Letter (S) are not Significantly Different (P> 0.01)**

Separate fungal pathogens were identified from infected greenhouse tomato and cucumber plants.

In this stage, *P. aphanidermatum* (PA-Cu-P-V_1) was separated and identified from infected plants.

DISCUSSIONS AND CONCLUSIONS

The results of this study showed that, the use of *T. flavus* bioformulations containing chemical stabilizers such as sodium nitrate, carboxymethyl-cellulose, D-cycloserine, and magnesium sulphate reduced Pythium root rot significantly in both tomato and greenhouse cucumber. No accurate information could be obtained about the use of additives in increasing the durability of biological compounds. But according to the research done, increased the efficiency and sustainability of biological compounds is crucial for their marketing and commercialization (Kaewchai *et al*., 2009; Mukhopadhyay and Maiti, 2009; Ghaderi-Daneshmand *et al*., 2012).

Total chemicals, including salts (sodium nitrate, potassium phosphate, and magnesium sulphate), amino acids like L-asparagine, and sugars like L-sorbose, have been introduced in the form of specific mediums as growth promoters of important fungal soil-borne pathogens, such as Verticillium and Fusarium (Katan and Ovadia, 1975; Christen, A. 1982; Hadar and Katan, 1989). However, research also showed that each of the above-mentioned chemicals alone and at certain concentrations inhibited the factors' growth (Veverka *et al*., 2007). In the present study, the results showed that the *T. flavus* bioformulations containing sodium nitrate was effective in controlling the tested soil-borne fungal diseases (tomato and greenhouse cucumber Pythium root rot). Thus, according to the above explanations, it was not unexpected.

On the other hand, an osmotic stabilizer such as sodium nitrate was reported to be a stabilizing compound for the enzyme chitinase (Gavanji *et al*., 2013; Patil and Jadhav, 2015). Therefore, such a compound could play a major role in keeping the metabolite related to the mechanism of *T. flavus* micro-parasitism, which is also chitinase (Inbar and Chet, 1995). So, *T. flavus* bioformulations treated with the above-mentioned metabolite have also shown optimal efficiency in controlling the diseases studied.

In this study, regarding the effect of two different *T. flavus* bioformulations containing the stabilizers of carboxymethyl-cellulose and D-cycloserine on the diseases studied, different results were observed for tomato and greenhouse cucumber. For tomato, the *T. flavus* bioformulation containing carboxymethyl-cellulose acted well in controlling the disease compared with that containing D-cycloserine. But for cucumber, this was not so. To interpret this, some remarks about the relationship between the effective metabolites of the different *T. flavus* isolates in terms of control pathogens and the host plant are essential. These are as follows:

Among the non-volatile metabolites of *T. flavus*, including glucose oxidase enzyme, gesilosidase, and beta-galactosidase, glucose oxidase plays a major role in controlling some plant pathogens (Jat and Agalave, 2013). The enzyme activity occurs in the presence of the glucose present in the root secretion of the host plant. By producing the toxic compound hydrogen peroxide, it can destroy different pathogenic fungi and bacteria (Kim *et al*., 1989). So, the enzyme could have acted more actively for *T. flavus* isolates, around the roots of plants with the root secretion rich in the available sugar compounds. Mutually, in *T. flavus* isolates obtained from the rhizosphere, the amount and activity of glucose oxidase metabolite will be significantly greater compared with the *T. flavus* isolates of plants with poor root secretion of sugar compounds.

In the present study, *T. flavus* isolates related to each plant (tomato or greenhouse cucumber) were used for same plant. Therefore, according to the above-mentioned information, it can be concluded that due to the presence of more sugar compounds in the rhizosphere of tomato than that of cucumber, the enzyme glucose oxidase in the isolate related to tomato was more active, and available sugar compounds, such as carboxymethyl-cellulose, were used. On the other hand, both D-cycloserine and carboxymethyl-cellulose compounds are considered as stabilizers for non-volatile compounds (Matos *et al*., 2012). Thus, for *T. flavus* isolate related to greenhouse cucumber (a plant with poor root secretion of sugar compounds), D-cycloserine was effective in controlling pathogens by increasing the sustainability of the other non-volatile compounds studied, including beta-galactosidase and gesilosidase. So, it can be concluded that the *T. flavus* bioformulations containing the stabilizer sodium nitrate had the optimal efficiency in the control of Pythium root rot in tomato and greenhouse cucumber.

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